AN ELECTRON MICROSCOPE STUDY OF MATERIAL FROM TISSUE OF THE CENTRAL NERVOUS SYSTEM OF POLIOMYELITIC AND NORMAL MICE AND COTTON RATS

MORRIS RHIAN*, SERGE G. LENSEN* AND ROBLEY C. WILLIAMS**

Received for publication May 16, 1949

I. INTRODUCTION

Our knowledge of the physical and chemical characteristics of the virus of poliomyelitis is attended by a considerable degree of uncertainty and contradiction, despite the great amount of work that has been done to describe these characteristics with precision. Ultrafiltration studies have indicated that the viruses of the poliomyelitis group have a size between 8 and 17 mμ (1, 2). Several workers in the field of electron microscopy have described what they believe to be the virus of poliomyelitis, but, in our opinion, there is no convincing evidence to date that the virus has been identified on electron micrographs. It is the purpose of this paper to present evidence to show why there is reasonable doubt that the virus of poliomyelitis has been identified by electron microscopy.

Gard (3, 4) has worked extensively with the viruses of mouse encephalomyelitis (Theiler's virus) and human poliomyelitis, and has reported certain physical characteristics based upon ultracentrifugation and electron microscopy.*** His starting materials and purification procedures varied greatly during his experiments, but his general conclusions were that the viruses of the poliomyelitis group are rod-shaped, with a size 15 × 115 mμ. His published electron micrographs are difficult to interpret, particularly since rods similar to those seen in infective material were also present in preparations from noninfective sources. Also, Gard states that numerous round bodies were seen in earlier stages of purification in preparations from both infective and noninfective material. Furthermore, Gard reported evidence for aggregation of what were assumed to be the basic sized virus particles.

There are several additional sources of uncertainty in the work of Gard which make it difficult to evaluate his electron microscopy. Gard states that some of his purified preparations had been stored in the laboratory for over a year before

*Division of Laboratories, Michigan Department of Health, Lansing, 4.

**Department of Physics, University of Michigan, Ann Arbor.

***The following facts concerning the material shown in Gard's micrographs should be carefully considered in any attempt to interpret them. He states in his paper (3, p. 41) that "The material was not sufficiently large to permit analysis of each preparation separately, so two pools were prepared, one (I) of murine specimens (1, 4a and 4c), the other (II) of human specimens (2, 3a, 3b, and 3c)." The separate speciments in each of the two categories were greatly different in their histories, purities, infectivities and sources. The size distribution curves range from lengths of 100 to 500 μ, with all lengths over 500 mμ arbitrarily excluded from the analysis. Further, a correction was apparently made for what Gard believed to be signs of stretching of the fibers; otherwise the histograms shown in his paper clearly indicate that the mean length of the particles is about double that reported.

examination in the electron microscope. Some of the highly purified preparations were not tested for infectivity and most of those known to be infective were also known to contain impurities. All of the specimens he observed in the electron microscope were suspended in distilled water, a procedure which we have found to cause agglomeration of particles.

Melnick (5) repeated some of Gard's purification methods, and observed rod-shaped particles similar to those reported by Gard, in material prepared from stools of normal and poliomyelitic humans. Melnick noted, however, that in the preparations observed in the electron microscope there were numerous round bodies in addition to the rod-like forms.

Both Gard's and Melnick's pictures exhibit the poor contrast characteristic of non-shadow-cast preparations of small particles of biological material. Any attempt at a strict interpretation of their micrographs is, therefore, difficult.

The development of the shadow-casting technique by Williams and Wyckoff (6) has made possible an enhanced precision in the observation of small biological particles, owing to an enormous increase in contrast and to a greater elucidation of three dimensional structure. Loring, Marton and Schwerdt (7) were the first to report the use of the new technique in the study of material from poliomyelitic sources. They observed that in their electron micrographs of preparations purified by differential centrifugation from tissues of cotton rats infected with the Lansing strain, there were several fields containing nearly spherical particles of moderately varying sizes, but averaging 25 mμ in diameter. Loring and his associates (7, 8) conclude that the micrographs exhibit the presence of almost pure poliomyelitis virus from the following considerations: (a) the differentially centrifuged preparations were essentially mono-disperse when subsequently examined in an analytical rotor; (b) the concentrated suspensions had a high infectivity titer; (c) normal material when treated the same way yielded 5-20 per cent of the total nitrogen contained in the infective material after purification. They did not, however, (in contrast with Gard and Melnick) examine their similarly treated normal material in the electron microscope, nor did they examine it in the analytical ultracentrifuge. Beard, in a recent paper, (9) has questioned the soundness of the conclusions of Loring, Marton and Schwerdt concerning the degree of purity of their preparations.

Gollan and Marvin (10) have reported on the electron microscopy of material purified by alcohol precipitation from CNS tissue of mice infected with the MM strain of poliomyelitis virus. They state that micrographs of such material exhibit particles ranging from 10 mμ to 20 mμ in diameter. However, they advance no opinion as to the degree of relationship between the particles photographed and the MM strain of virus, and they report no comparisons with uninfected material.

We have presented a preliminary report of our attempts to identify the virus of the Lansing strain of poliomyelitis from mouse material (11). The present report describes this work in detail, together with the results of more recent work on cotton rat material, undertaken in order to ascertain whether micrographs of the purified preparations vary with the species of animal from which the tissue is taken and with different purification procedures.

II. EXPERIMENTAL

During the past three years we have examined by electron microscopy suspensions of material obtained by differential centrifugation from tissues of the central nervous system of mice and cotton rats. Wholly comparable material from both normal (noninfected) and poliomyelitic animals has been subjected to the same treatments and examined at the same time throughout this work. The suspensions were derived from 22 independent sets of source material of both normal and infected tissue which were carried through the purification procedures, were tested for biological activity, and were examined in the electron microscope.

In the preparative work with tissues from mice the following procedure was routinely used: The mouse adapted Lansing strain of poliomyelitis virus was used. White Swiss mice about two weeks old were inoculated intracranially with 0.03 ml of a 10 per cent suspension of infected spinal cord and medulla tissue. Those mice which showed unmistakable signs of paralysis in at least two legs, within six days after inoculation, were sacrificed for stock virus material.

The spinal cords and medullae of the sacrificed mice were removed under aseptic conditions and stored in a freezer at either −55 to −60 C, or at −30 C. From portions of the frozen stock material, thawed just before use, 10 per cent suspensions of the tissue were prepared. Some of the suspensions were prepared by grinding with sterile alundum in a mortar with pestle, and others by fragmenting with a Waring Blendor. Buffered saline solution*, pH 7.4, was used as the suspending medium in all cases.

The crude 10 per cent suspension was cleared of gross particles by centrifuging in a horizontal carrier at approximately 800 g for 25 minutes. The supernatant suspension was further clarified by centrifugation at *ca* 29,000 g for 15 minutes. The sedimented material was then discarded, and the clear supernate used for further purification. It was established that there was no observable loss of titer accompanying this clarification procedure.

To obtain sedimentation of the virus, the clarified suspension was centrifuged in a Masket type rotor for three hours at *ca* 120,000 g. The supernate (which had been shown to contain less than 1 per cent of the total virus activity of the original suspension) was pipetted or poured off and the tubes drained. The sedimented material was resuspended in, and the tubes rinsed with, successive portions of saline solution by mixing with a pipette. These suspensions and washings were pooled in a 100 ml Pyrex centrifuge bottle and the volume adjusted to that of the original 10 per cent suspension. This suspension was mixed by vigorous mechanical shaking for 15–20 minutes. In two instances powdered alundum was used as an aid in dispersing the material during shaking, but in all other cases no material of this kind was used. After the shaking the undispersed matter was removed by centrifuging in an angle head at *ca* 3200 g for 15 minutes. This cycle of differential centrifugation (pelleting of the virus, resuspension and clearing) was repeated two or three times as desired. After each treatment the volume of the suspension was adjusted to correspond to the original 10 per cent tissue suspension. Titrations of all fractions separated in this procedure established that there was no significant loss of virus activity during two cycles of centrifugation (16). Losses were usually large during subsequent centrifugation treatments.

---

* Physiological saline contained 0.15M sodium chloride plus 0.02M sodium phosphate at pH 7.4.

In one instance the pellet after the second sedimentation was resuspended in distilled water. Much material was separated during clarification of this suspension and only little virus activity was retained in the water suspension.

Suspensions used for electron microscope examination were in some instances subjected to further clarifying procedures in an effort to obtain a suspension of particles of uniform size. These treatments involved longer periods of centrifugation at low speed and/or short periods of centrifugation at high speeds (the latter corresponding to the procedure of Loring and Schwerdt (8, 12)). Since none of these processes produced any marked change in the electron-microscopic appearance of the suspensions the details are not given, but some specific examples are stated in the legends of the figures.

An attempt was made to demonstrate a specific effect, possibly observable in the electron microscope, by treating suspensions of mouse virus, purified by the above procedure, with antiserum of known titer against the Lansing strain of virus. The neutralization test was carried out by using varying amounts of serum and a constant (1 per cent) dilution of the virus suspension. The end point of the neutralization test is the calculated dilution of serum which allowed 50 per cent survival of the infected mice. Duplicate portions of the serum virus mixtures were examined in the electron microscope for evidence of agglutination or other changes in a specific component of the mixtures.

During the work on the cotton rat tissue, three attempts were made to repeat the purification procedure reported by Loring and Schwerdt (8, 12). This technique was revised for a fourth experiment to include a further unpublished modification in the above procedure (13). This change was the replacement of clarification of the redispersed pellet suspension at 67,000 g for 5 minutes by clarification at 2700 g for 20 minutes. A variation between the procedures published and modified (8, 12, 13) was that our tissues were stored at approximately −30 C in mechanical freezers instead of at −35 to −60 C in a dry-ice chest as reported by Loring and Schwerdt.

Samples of all suspensions examined in the electron microscope were checked for virus activity by determining the minimum infective dose as the 50 per cent endpoint calculated by the method of Reed and Muench (14). For these titrations ten serial dilutions were inoculated into groups of six mice or five cotton rats and the animals observed daily for 21 days. The LD_{50} is routinely expressed as the percent concentration of the original tissue. This has the advantage of being independent of some arbitrarily chosen reference such as "virus nitrogen" and allows direct comparison of the titers of all fractions without reference to their chemical composition. An LD_{50} of the order of 0.05 per cent tissue concentration (1:2000 dilution) represents a typical satisfactory titer in our experience.

It was established that the material prepared from tissues of normal animals was free from active Theiler's mouse encephalomyelitis virus and other infective agents. This was done by intracranial injection of 10 per cent tissue suspensions in groups of mice and cotton rats and observing them for not less than 20 days.

Some of the purified suspensions were analyzed for total nitrogen by a micro-Kjeldahl method (15). On the basis of the N content of the suspensions, the expressions of some of the titers were converted to grams of nitrogen to facilitate comparison with data in the literature.

For the electron microscope examinations serial dilutions of the purified suspensions were prepared in saline, except for one instance when distilled water was used. A drop of the suspension from a capillary pipette was allowed to dry on the collodion covered microscope specimen screen. The salts remaining after drying were removed by flooding the screen with a large drop of water which was removed after a few seconds with a capillary pipette. After the screens were again dried they were treated by the shadow-casting technique (6). In most instances gold was used as the deposited metal at a calculated thickness of 8A. Uranium was used in the preparation of some of the screens, including all those made from cotton rat material. The metal was deposited at a 7:1 angle in our early work, but subsequently a 5:1 angle has become routine.

Several screens and many fields on each screen were examined for each suspension photographed. Micrographs which were judged to be typical of the many fields examined were taken at direct magnification of about × 10,000. In the examination of the material from the suspensions of normal material, fields were photographed which were typical of the suspension, as well as fields which were most similar in appearance to the corresponding preparations from the infected tissue. Usually the same field fulfilled both criteria.

In all this work every effort was made to have the conditions uniform throughout any series of experiments. The total elapsed time during any purification procedure was kept at a minimum and never exceeded 60 hours. All suspensions were kept cold except when actually being handled, and exposure to room temperature was kept as brief as possible. Portions of the purified suspensions were prepared for the electron microscope examination at the same time that duplicate portions were being used for the titrations. Aseptic technique was used at all times except in the preparation of the microscope screens.

III. RESULTS

A. Electron Micrography

From a total of 235 micrographs we have chosen those shown in figures 1-10 as typical of the mouse material, and those shown in figures 11–14 as typical of the cotton rat material. Figure 1 shows a quite uniform dispersion of round, probably spherical, particles with an average diameter of 25–30 mμ. The purified suspension from which this screen was prepared had an LD_{50} of 0.31 per cent. In addition to the dispersion of spherical particles this micrograph shows in the upper left hand corner small objects later found to be due to some material leached out of the walls of lime glass bottles. These particles are not seen in pictures taken following the use of saline solution prepared in Pyrex flasks.

Figure 2 is another picture of the same suspension. The particles here seem to be more irregular in shape than those shown in figure 1, but this may be due to their greater dispersion over the screen used in the microscope. (These two figures illustrate the great variation that can be encountered in examining two specimens from the same suspension).

Figure 3 is of a preparation from a highly infectious suspension ($LD_{50}=0.013$ per cent), shadow cast with about 6A of uranium, and displaying a dispersion of very small particles, along with larger flat masses. The round, probably spherical, particles have a diameter of 6–10 mμ. Such small particles probably would not be distinguishable without the aid of shadow-casting.

Figure 4 shows a dispersion of isolated particles of approximately 15 mμ diameter. Although there was high infectivity in the partially purified stock suspension, the further clarified suspension illustrated here had lost its virus activity. There is no way of determining whether the objects seen are inactivated virus particles or are wholly unrelated to the virus.

Figure 5 illustrates an aggregation of particles from poliomyelitic suspensions which, when first observed we considered to be characteristic of the virus inasmuch as we failed for a period of months to find these aggregates in suspensions from normal tissues. The aggregates were later found, however, in non-infective suspensions from poliomyelitic tissue and in some control suspensions. Figure 6 (from inactivated material) is representative of those suspensions in which both aggregated and isolated particles are found to exist together.

Figure 7 illustrates the type of particles commonly found in the suspensions from normal tissues and exhibits a dispersion of isolated particles of varying size, *including all sizes found in the active and inactivated poliomyelitic suspensions.* Figure 8, also of control material, shows sharply outlined aggregates similar in appearance to those shown in figure 5.

Figure 9 is an example of a preparation obtained when the purified suspensions were diluted in distilled water in the preparation of the screens for the microscopic examination. Without the aid of shadow-casting such linear aggregates might easily be described as rod-like forms.

Figures 11–14 are typical of the preparations we obtained when cotton rat tissue was purified according to the procedures of Loring and Schwerdt (8, 12, 13). Figure 11 is of material obtained from poliomyelitic cotton rat tissue after the second cycle of centrifugation. The preparation is obviously far from homogeneous. The picture is characterized chiefly by the presence of large aggregates of material, and some separate particles which seem to be spherical. The particles are most clearly seen on top of the plate-like formations, but can also be distinguished in the areas between the larger formations.

Figure 12 is a typical preparation of material obtained from normal cotton rat tissue after treatment identical to that given to the material shown in figure 11. The aggregates and isolated particles are quite similar in appearance to those of figure 11.

Figures 13 and 14 are micrographs of material obtained from infected and

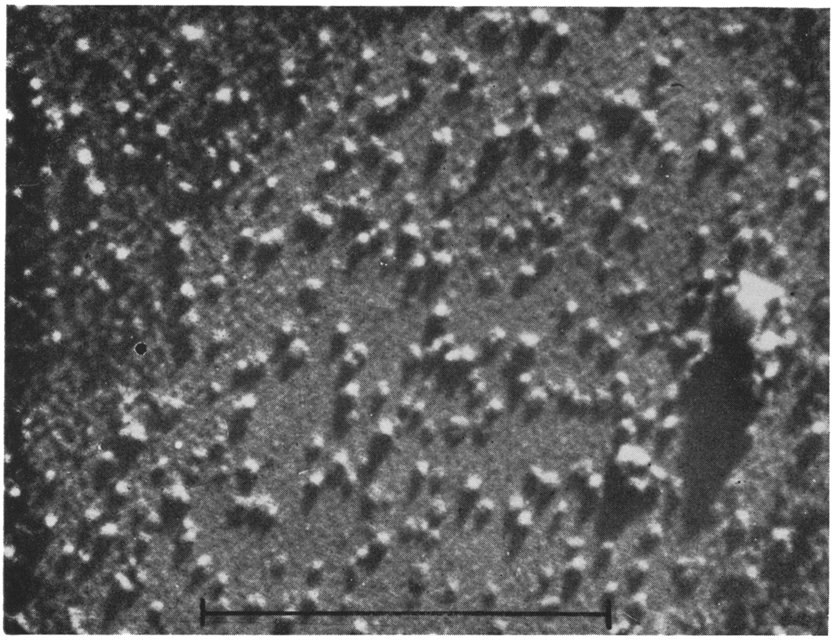

Figure 1. Active Preparation from Infected Mouse Material

Prepared by two cycles of centrifugation including clarification at 67,000 g for ½ hour after ach sedimentation and redispersion. The scale shown on this and the following figures indiates 1μ

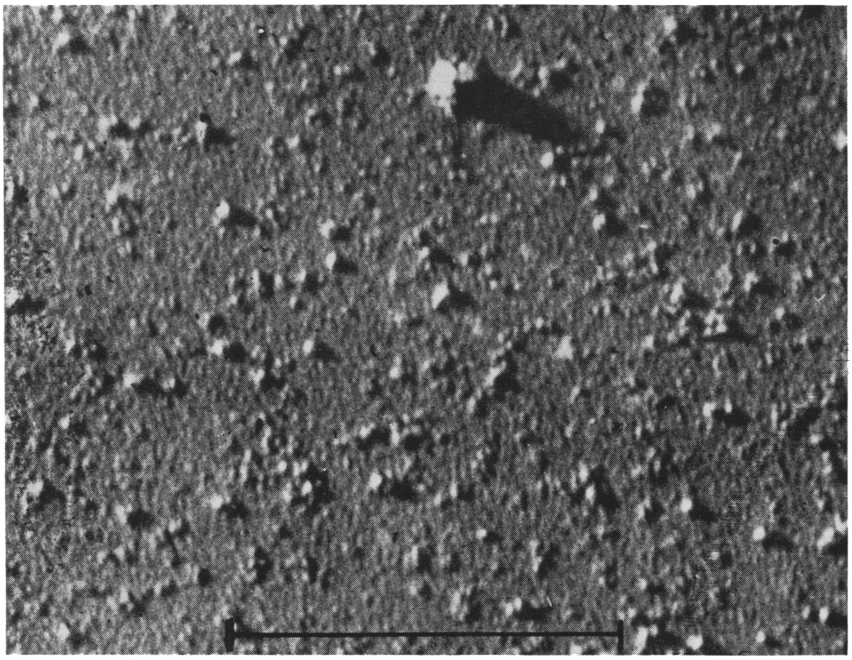

Figure 2. Same Material as in Figure 1, from Another Specimen Screen.

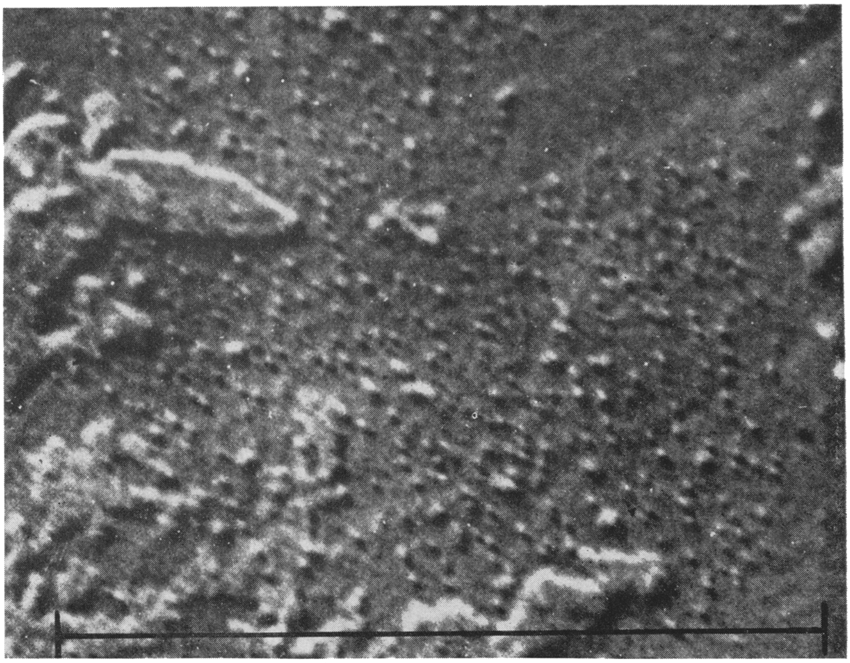

FIGURE 3. ACTIVE PREPARATION FROM INFECTED MOUSE MATERIAL
Prepared by one cycle of centrifugation.

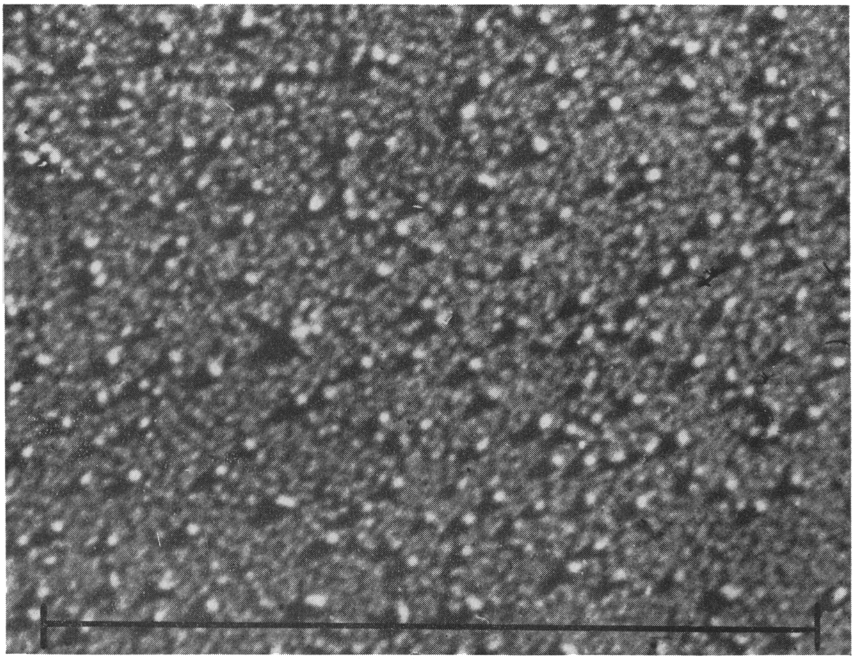

FIGURE 4. INACTIVATED PREPARATION FROM INFECTED MOUSE TISSUE
Prepared by three cyles of centrifugation plus additional clarification at 120,000 g for 10 minutes.

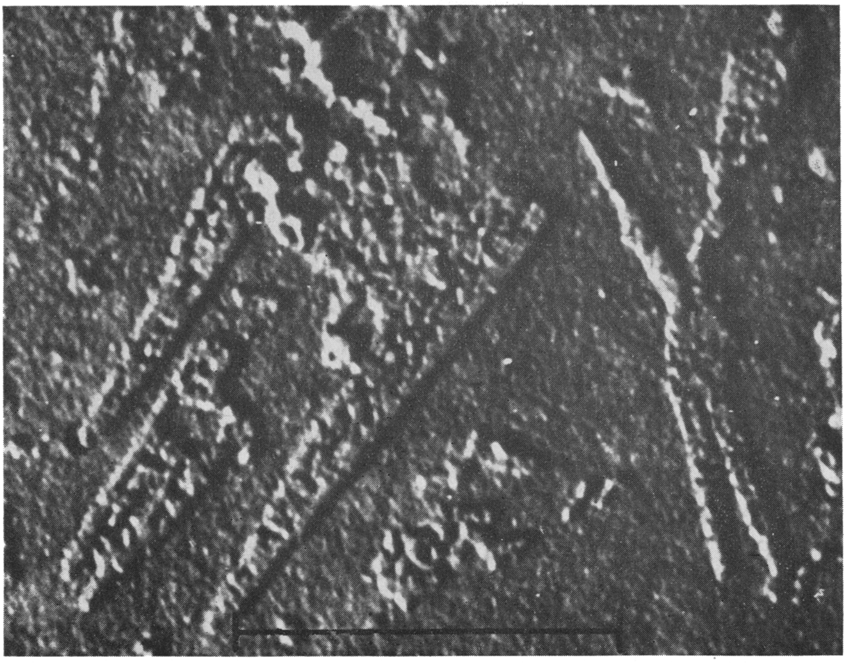

FIGURE 5. ACTIVE PREPARATION FROM INFECTED MOUSE TISSUE

Prepared by two cycles of centrifugation plus additional clarification runs, at 29,000 g for ½ hour followed by 67,000 g for ½ hour.

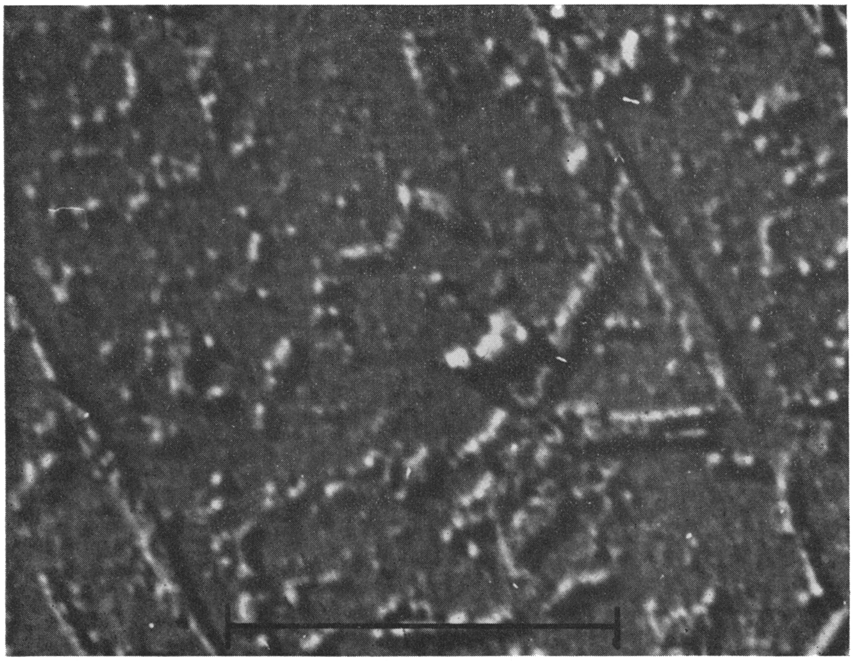

FIGURE 6. INACTIVATED PREPARATION FROM INFECTED MOUSE TISSUE

Prepared by two cycles of centrifugation followed by an additional clarification at 120,000 g for 10 minutes.

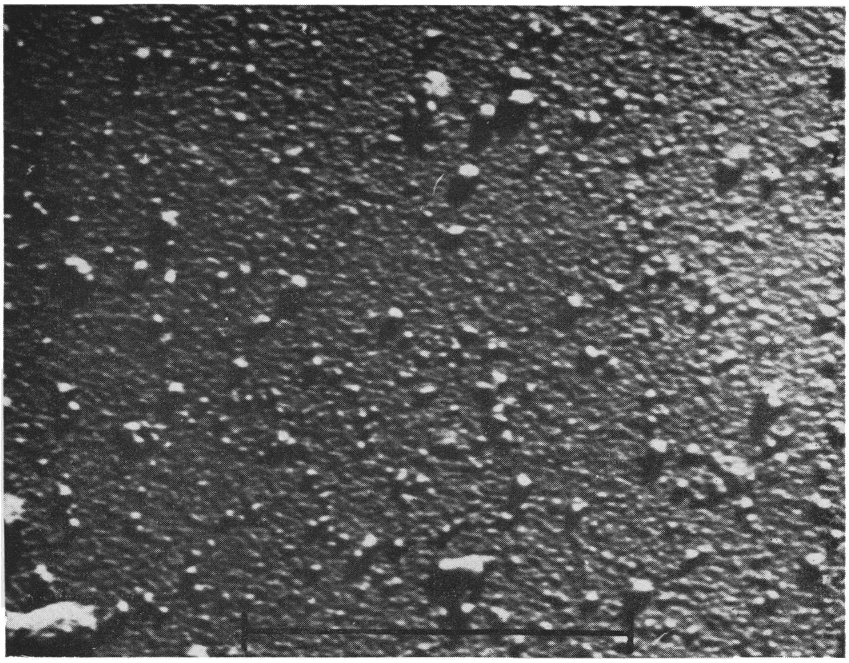

FIGURE 7. PREPARATION FROM NORMAL MOUSE TISSUE
Prepared by the same procedure as material for figure 6.

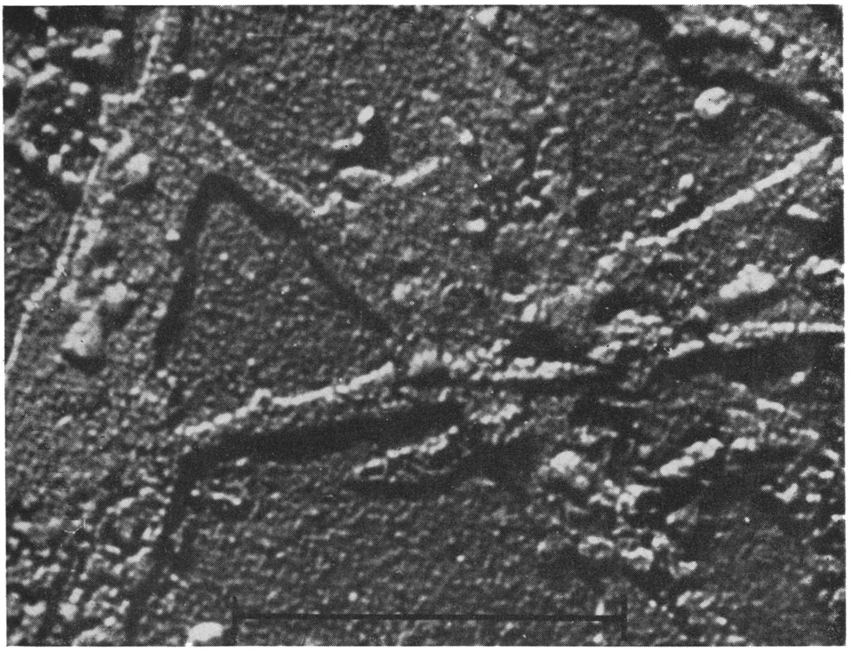

FIGURE 8. PREPARATION FROM NORMAL MOUSE TISSUE
Prepared by three cycles of centrifugation and clarified at 29,000 g after third cycle.

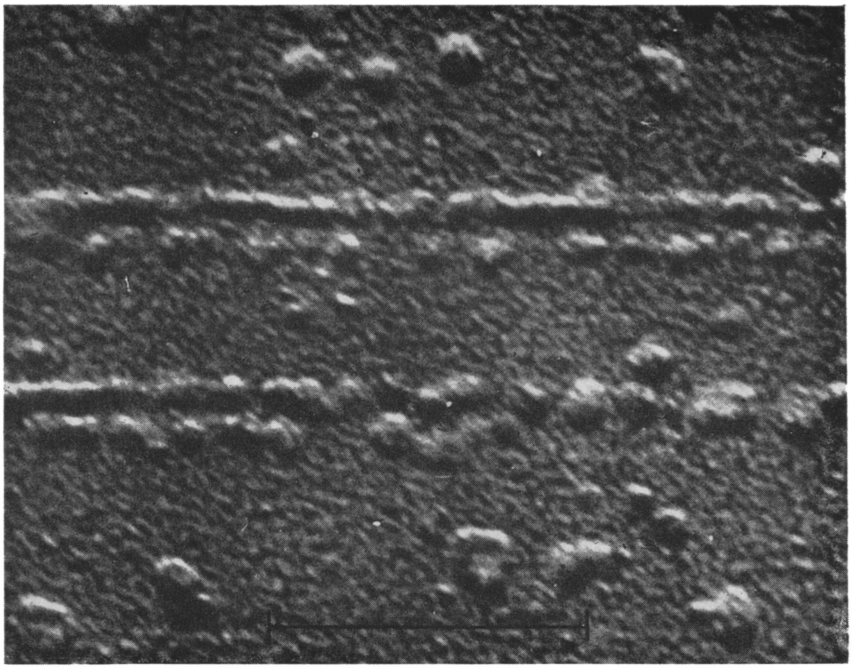

FIGURE 9. PREPARATION WITH LOW VIRUS ACTIVITY FROM INFECTED MOUSE TISSUE
Distilled water was used as diluent after second sedimentation. Specimen diluted in distilled water to prepare mount for electron microscope.

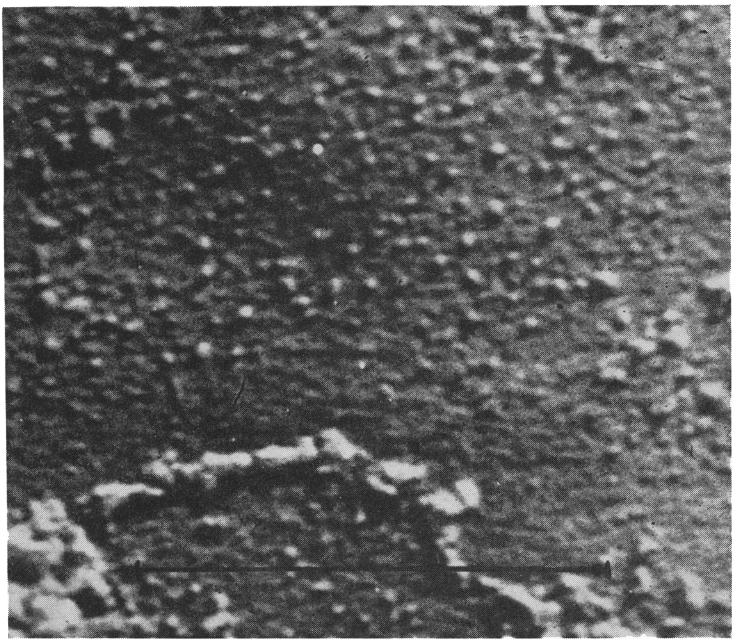

FIGURE 10. PREPARATION FROM NORMAL MOUSE TISSUE
Prepared by one cycle of centrifugation.

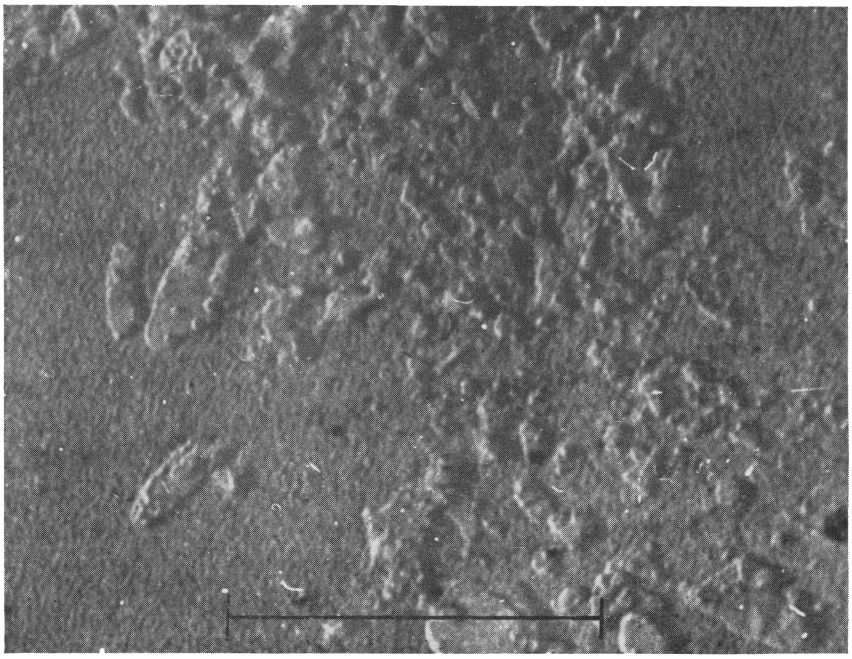

FIGURE 11. PREPARATION FROM INFECTED COTTON RAT TISSUE
No virus activity in mice. Prepared by two cycles of centrifugation according to procedure published by Loring and Schwerdt.

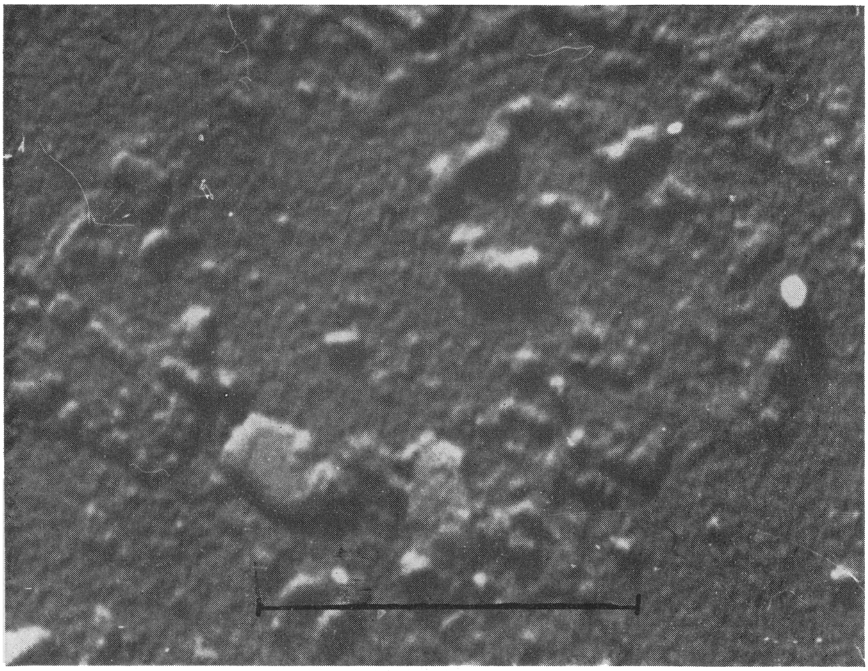

FIGURE 12. PREPARATION FROM NORMAL COTTON RAT TISSUE, TREATED IN SAME MANNER AS MATERIAL IN FIGURE 11.

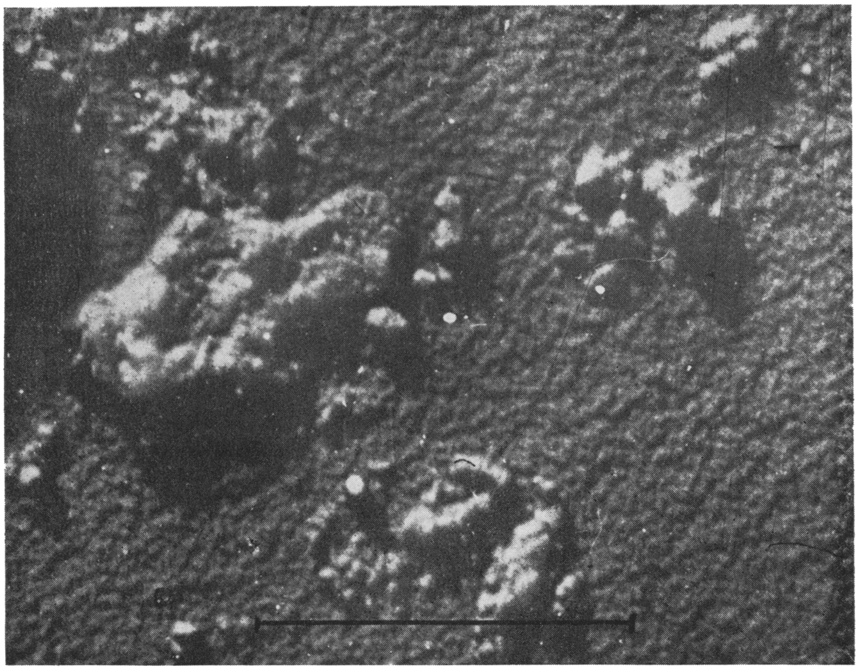

FIGURE 13. PREPARATION FROM INFECTED COTTON RAT TISSUE, WITH LOW VIRUS ACTIVITY, LD_{50} = 0.28 PER CENT FOR COTTON RATS

Prepared by one cycle of centrifugation, same procedure as for figure 11.

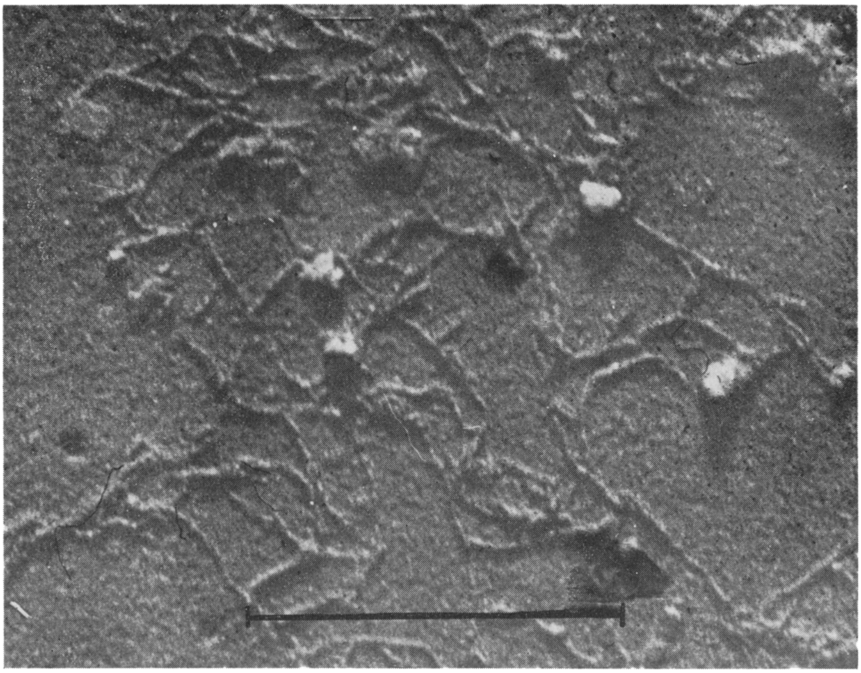

FIGURE 14. PREPARATION FROM NORMAL COTTON RAT TISSUE

Same procedure as figure 11.

normal cotton rats, respectively, after one cycle of centrifugation. The form of the aggregates is different from those of figures 11 and 12 and there are fewer isolated particles. In figure 14 the form of the aggregates is such that in an unshadowed preparation they might appear as filaments or long rods.

When particles shown in the micrographs are judged on the bases of uniformity of size, uniqueness of shape, or specific occurrence, it is apparent that all the preparations we have obtained by differential centrifugation are far from homogeneous. In addition, we have been unable to associate high virus activity with any of the above criteria of purity or unique occurrence.

TABLE 1

Nitrogen Content of Suspensions from Normal and Poliomyelitic Cotton Rat Tissue at Various Stages in the Purification Procedure

STAGE OF PURIFICATION	RUN NO.	SOURCE		INFECTIVITY LD_{50}, g N
		Normal mg/ml	Infected mg/ml	
Clarified 20 per cent tissue suspension	4	1.027	1.088	$10^{-7.59}$
Above, after ether extraction	4	0.871	0.789	
Frozen, stored (27 days), thawed, clarified suspension	4	0.351	0.339	$10^{-7.49}$
	3	0.345	0.356	$10^{-7.15}$
	2	0.440	0.482	
	1	0.408	0.528	
Average..................................		0.386	0.426	
After first cycle purification	4	0.015	0.017	$10^{-9.26}$
	3	0.010	0.010	$10^{-7.77}$
	2	0.012	0.016	
	1	0.015	0.017	
Average..................................		0.013	0.014	
After second cycle purification	4	0.009	0.011	$10^{-8.28}$
	3	0.002	0.001	**
	2	*	*	
	1	*	*	

* Nitrogen content too low for determination in the sample available.

** Endpoint not determined, above 2 per cent solution.

The attempt to recognize by electron microscopy a reaction between the neutralizing serum and a specific component of the purified virus suspension was without success. The 50 per cent endpoint of the convalescent serum was a 1:500 dilution. However, there was no difference in the appearance in the microscope of the mixture of virus suspension and neutralizing serum and a mixture of virus suspension plus normal horse serum.

B. Nitrogen Yields

The yields of nitrogen obtained from our purification of mouse material have been reported elsewhere (16). They are of the order of 0.4 mg nitrogen per gram of original tissue in one cycle preparations, 0.17 mg in second cycle preparations and 0.01 mg per gram in third cycle preparations. Our yields of nitrogen from the cotton rat tissue are summarized in table 1. In the first two runs the total yield of material was too low to allow determination. In the second cycle preparation of the third run the yield was 0.02 mg nitrogen per gram and 0.01 mg per gram from the normal and infected tissues, respectively.

IV. DISCUSSION

The results described and illustrated above emphasize a number of difficulties encountered in attempts to separate, and subsequently to identify by electron microscopy a characteristic particle having a specific biological activity. As shown in our micrographs a practically continuous range of sizes of particles is generally obtained by differential centrifugation from normal tissue of the central nervous system. It has yet to be demonstrated that differential centrifugation, at least as ordinarily practiced, is selective enough to allow complete separation of particles of closely limited ranges of size, as shown by electron microscopy.

Our work confirms the observations of Gard and of Melnick that particles of similar appearance are obtained without exception from both normal and infected tissues by differential centrifugation, and confirms the report of Loring, Marton and Schwerdt that no rod shaped or filamentous forms are obtained from tissues infected with the Lansing strain of poliomyelitis virus. We have been unable to confirm by the evidence of electron microscopy the statements of Loring and Schwerdt that poliomyelitis virus of 80 to 95 per cent purity (that is, containing from 5 to 20 per cent of non-virus material) can be separated from the infectious tissue, and that the particles of uniform size in their preparations can be identified as poliomyelitis virus. Neither have we noted that a significantly greater yield of nitrogenous matter can be obtained from the infected tissue than from normal tissue.

The amount of material obtained in the final purified preparations from normal and poliomyelitic tissues has always been reported to be very low. Gard reported (3) the average yield of material to be 0.6 micrograms per mouse brain. Loring and Schwerdt report (8) their average yield as 0.1 to 0.2 mg. of "virus nitrogen" per 100 grams of starting material. Our yields of total nitrogen have been much higher than this, (1–10 mg/100g) in preparations having high titers.

Loring and Schwerdt have reported marked differences in the amount of nitrogenous material obtained from normal and infected tissues. In particular, they reported a marked precipitation of supposedly normal protein attendant upon freezing and thawing the ether-extracted, clarified suspensions of tissue. It is not apparent from their publications that normal and infected tissues were treated under *identical* conditions at the *same* time. We have observed the precipitation of as much as 57 per cent of the total nitrogen attendant upon freezing and thawing. However, we have noted no significant difference between the normal and infected tissues in this respect, when both were treated at the same time under the same conditions. As shown in table 1 the average yield of total nitrogen in our suspensions after freezing is less than 10 per cent greater in the infected than in the normal suspensions.

The procedure for handling the cotton rat material in the fourth run resulted in a greater retention of nitrogenous material in the second cycle preparations (table 1). With this procedure the loss in titer was about 10-fold but total nitrogen decreased only about 15 per cent between the first and second cycle virus suspensions.

There is one general difference in the technique used by Loring and Schwerdt and that used by us inasmuch as they apparently concentrated (by volume reduction) as well as purified all their preparations. We have attempted only to purify our preparations at a constant volume. Our first three attempts to use the purification procedure published by Loring and Schwerdt resulted in such great losses of virus activity that an endpoint could not be obtained after starting the titration with a 2 per cent solution of the material. Presumably, if we had been working with greater amounts of starting material, and had concentrated the suspensions as much as 150 times during the purification procedure, we might have experienced as great losses of material and still retained enough virus to yield a high titer based on the nitrogen content of the purified preparation. From the data of our fourth trial, for example, it is seen that relatively little purification is obtained by repeated cycles of centrifugation. The ether extracted suspension of the infected tissue had an end-point titer of $10^{-7.59}$ g of nitrogen. After freezing, storing, thawing and clarifying the suspension had a titer of $10^{-7.49}$ g nitrogen. These figures compare with titers of $10^{-6.5}$ and $10^{-7.9}$ g nitrogen reported by Loring and Schwerdt for comparable preparations. After the first cycle of differential centrifugation the titer of our suspension rose to $10^{-9.26}$ g of nitrogen and after the second cycle it fell to $10^{-8.28}$. This would indicate that repeated centrifugation removed both normal and virus protein in nearly the same ratio in which they exist in the suspension.

It is difficult to interpret the data published by Loring and Schwerdt in 1942 (12) because monkeys were used as the test animals, and it was not feasible to get a sharp titration of the virus activity of the suspensions. Also, a comparison of the yields of nitrogenous material from the normal and infected tissues is difficult because, as reported in their table III (12) for the case of the frozen (nonglycerolated) samples, the periods of storage and degree of purification of the normal and infected tissues were not the same in any instance.

The later data of Loring and his associates (8) on the yield of nitrogenous material and virus activity in the preparations, as related to time of storage in the frozen state, show that such storage has little effect on the specific activity of the suspensions. As stated in their table I (8) the titer of a typical suspension before freezing was $10^{-6.5}$ g of nitrogen. After storage for four days in the frozen state there was a 25 per cent decrease in total soluble nitrogen and a titer of $10^{-5.9}$ g. After storage of 45, 65 and 120 days the yields of soluble nitrogen were

55, 51, and 53 per cent, while the corresponding titers were $10^{-7.9}$, $10^{-7.3}$, and $10^{-7.2}$ g of nitrogen.

No comparable data concerning N precipitation from normal tissue are given. If a significant change in titer of the suspension were to occur, and be due solely to the precipitation of normal protein from the virus suspension, one would expect the total nitrogen to decrease to nearly 10 per cent of the original value instead of only to 50 per cent.

The most nearly pure preparations of poliomyelitis virus obtained by Loring and Schwerdt had LD_{50} titers of approximately 10^{-10} g nitrogen. It is interesting to calculate from these data the infective dose of this virus on the basis of number of particles involved. If we assume a sphere of an average diameter of 25 mμ as reported by Loring, Marton and Schwerdt to represent the virus particles, the average volume of the particles would be 8.2×10^{-18} ml. Using the average density of biological material (1.25 g/ml) the mass of each particle would be 1×10^{-17} g. From an average infective dose of 10^{-10} g of nitrogen (6×10^{-10} g protein) from the purified preparations we find that the dose would be equivalent to 6×10^{7} particles of the size assumed for the virus particle. This implies that either the virus is remarkably noninfectious or else it has been obtained at best in extremely impure form. Our electron micrographs would support the latter implication.

From the work reported here and from a careful consideration of the data previously published by others, we must conclude that until photographs of particles of fairly uniform size are obtained from infected material, and particles of similar size and shape are not photographed from uninfected material treated identically throughout preparation, the identification by electron microscopy of the virus of poliomyelitis is still an open problem.

V. SUMMARY

Electron-microscopic study of purified preparations, derived from central nervous system tissue of mice and cotton rats by differential centrifugation, has shown that particles alike as to size and shape are obtained from both normal and poliomyelitic tissue. The total yield of nitrogenous material has been essentially the same from both normal and infected tissues.

Characteristic particles separated from both normal and infected tissues are approximately spherical and in the size range from 6 to 30 mμ in diameter. Aggregates of the particles are often obtained.

No intact rods or long filaments have been detected in any of the preparations, but some of the aggregations could easily be mistaken for such forms in unshadowed micrographs.

From a critical examination of our work, and that of others, we conclude that there is no evidence that a virus of the poliomyelitis group has ever been unequivocally identified on electron micrographs thus far published.

REFERENCES

1. Elford, W. J., Galloway, I. A. and Perdrau, J. R. 1935 Size of virus of poliomyelitis as determined by ultrafiltration. J. Path. & Bact. **40:** 135–141.

2. Theiler, M. and Bauer, J. H. 1934 Ultrafiltration of virus of poliomyelitis. J. Exper. Med. **60:** 767–772.
3. Gard, Sven 1943 Purification of poliomyelitis viruses. Experiments on murine and human strains. Acta med. Scandinav. Suppl. **143:** 173 pp.
4. Gard, Sven 1943 Uebermikroskopische Beobachtungen an gereinigten Poliomyelitis-Viruspräpareten. III. Ein Vergleich mit den physikalischchemischen Versuchsergebnissen. Arch. ges. Virusforsch. III. 1–17.
5. Melnick, J. L. 1944 Detection with electron microscope of rod-shaped particles in stools of normal and poliomyelitic individuals. J. Immunol. **48:** 25–28.
6. Williams, R. C. and Wyckoff, R. W. G. 1945 Electron shadow-micrography of virus particles, Proc. Soc. Exper. Biol. & Med. **58:** 265–270.
7. Loring, H. S., Marton, L. and Schwerdt, C. E. 1946 Electron microscopy of purified Lansing virus. Proc. Soc. Exper. Biol. & Med. **62:** 291–292.
8. Loring, H. S. and Schwerdt, C. E. 1946 Isolation of a macromolecular constituent with properties of the Lansing strain of poliomyelitis virus. Proc. Soc. Exper. Biol. & Med. **62:** 289–291.
9. Beard, J. W. 1948 Review. Purified animal viruses. J. Immunol. **58:** 49–108.
10. Gollan, Frank and Marvin, J. F. 1948 Electronmicroscopy of the purified MM poliomyelitis virus. Proc. Soc. Exper. Biol. & Med. **67:** 366–367.
11. Rhian, M., Williams, R. C. and Lensen, S. G. 1948 Electron microscopy of partially purified material from spinal cords and medullae of normal and poliomyelitic mice. Proc. of Meetings, Society of American Bacteriologists **1:** 78.
12. Loring, H. S. and Schwerdt, C. E. 1942 Studies on the purification of poliomyelitis virus. I. Yields and activity of preparations obtained by differential centrifugation. J. Exper. Med. **75:** 395–406.
13. Anderson, Jane C. 1948 Private communication from Dr. Loring's laboratory.
14. Reed, J. L. and Muench, H. 1938 Simple method of estimating 50 per cent endpoints. Am. J. Hyg. **27:** 493–497.
15. Umbreit, W. W. 1945 In manometric techniques and related methods for the study of tissue metabolism. Burgess Publishing Co., Minneapolis, Minn. p. 103.
16. Lensen, S. G., Rhian, M. and Stebbins, M. R. 1946 The inactivation of partially purified poliomyelitis virus in water by chlorination. J. Am. W. W. Assoc. **38:** 1069–1077